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Complete title
Iron handling by the human kidney: Insights from urinary iron and renal injury measurements in health and disease

Short title
Human renal iron handling in health and disease

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Abstract
Background: In physiological conditions, circulating iron can be filtered by the glomerulus, but is almost completely reabsorbed by the tubular epithelium to prevent urinary iron wasting. Increased urinary iron concentrations have been associated with renal injury, and may be the result of both increased glomerular iron filtration or insufficient tubular iron reabsorption. However, it remains uncertain whether both these processes actually contribute to urinary iron excretion and renal injury.
Methods: We measured plasma and urine iron parameters and urinary tubular injury markers in healthy human subjects (n=20), patients with systemic iron overload (n=20) and patients with various disorders of renal tubular dysfunction (n=18).
Results: Urinary iron parameters were increased in both patients with systemic iron overload and tubular dysfunction, whereas plasma iron parameters were only increased in patients with systemic iron overload. In patients with systemic iron overload, increased urinary iron levels associated with elevated circulating iron, as indicated by transferrin saturation (TSAT), and increased body as suggested by plasma ferritin. In patients with tubular dysfunction, enhanced urinary iron and transferrin excretion associated with distal tubular injury as indicated by urinary gluthathione s-transferase-pi-1-1 (GSTP-1-1) excretion. In systemic iron overload, elevated urinary iron and transferrin levels associated with increased proximal tubular injury, indicated by urinary kidney injury marker 1 (KIM1).
Conclusion: Our explorative study demonstrates that both glomerular filtration of elevated plasma iron levels and insufficient tubular iron reabsorption are associated with urinary iron excretion and renal injury.

Keywords
Iron, urine, filtration, reabsorption, injury

Introduction

In physiological conditions, circulating iron is bound to transferrin (transferrin-bound iron, TBI), which can be filtered by the glomerulus of the kidney into the renal tubular lumen (1). Subsequently, filtered TBI is suggested to be almost completely reabsorbed by renal tubular epithelial cells, since hardly any transferrin or iron is found in urine in healthy volunteers (1-3). In addition, micropuncture studies in rats demonstrated the presence of transferrin in primary urine and showed that iron reabsorption by both the renal proximal tubule (PT) and distal tubule (DT) decreased urinary ^{55}Fe excretion (4).

Increased iron exposure can be harmful for tubular epithelial cells, because iron is known to catalyze reactive oxygen species formation in the Fenton reaction and cause tissue injury (5). In human and porcine PT epithelial cells, iron overload exposure was shown to decrease cellular viability and proliferation and cause oxidative cellular injury (6-8). Moreover, increased urinary iron levels have been found in patients with nephrotic syndrome and diabetic nephropathy (3, 9-13), suggesting an association between enhanced urinary iron concentrations and renal injury. In animal models of renal diseases, such as minimal change nephrotic syndrome and nephrotoxic serum nephritis, increased urinary iron excretion coincided with renal tubular injury (14-16). Currently, it is not clear whether increased urinary iron concentrations in patients are the result of increased glomerular iron filtration and/or insufficient tubular iron reabsorption and if these processes contribute to renal injury.

During systemic iron overload, unrestricted iron intake from the intestine in HFE-hereditary hemochromatosis (HFE-HH) or additional frequent red blood cell transfusions in β-thalassemia major increase circulating TBI and also result in the presence of non-transferrin-bound iron (NTBI), when the iron binding capacity of transferrin is exceeded (17, 18). Moreover, iron accumulates in parenchymal tissues, like the liver and heart, represented by high plasma ferritin levels (17). In β-thalassemia major, plasma ferritin also associated with renal iron deposition (19). Increased urinary iron concentrations have been observed in patients with systemic iron overload (20-22), suggesting that glomerular filtration of increased circulating iron exceeds the tubular iron reabsorption capacity in these patients. Alternatively, urinary iron excretion can result from insufficient iron reabsorption in either PTs or DTs. Filtered proteins, including TBI (23, 24), are predominantly reabsorbed by PTs (25), but also DTs express transporters involved in iron handling and are reported to take up TBI (26, 27). Increased urinary transferrin levels in patients with Fanconi syndrome, characterized by compromised proximal tubular reabsorption (1), suggest the tubular reabsorption capacity affects urinary transferrin levels, and similarly, possibly also urinary iron levels.

To better understand the contribution of increased glomerular iron filtration and/or insufficient tubular iron reabsorption by PTs and DTs to urinary iron levels and related renal injury, we performed an explorative study and measured plasma iron parameters, urinary iron parameters and renal tubular injury markers among healthy subjects, patients with systemic iron overload and patients with various disorders of renal tubular dysfunction.

Materials and Methods

Study design

This explorative, observational study included subjects between November 2016 – October 2017. Healthy volunteers (n=20) were recruited in and around the Radboud university medical center (RUMC; Nijmegen, the Netherlands). Exclusion criteria included presence of renal disease, iron overload disorder or urinary tract infection, use of chelation medication or active menstruation. Patients with systemic iron overload disorders (n=20; Table 1) were included based on the diagnosis made by their treating physician and with most recent determined transferrin saturation (TSAT)>70% at RUMC and Amsterdam Medical Center (Amsterdam, the Netherlands). Patients with tubular dysfunction disorders (n=18; Table 1) were included based on the diagnosis made by their treating physician in RUMC, Erasmus Medical Center (Rotterdam, the Netherlands), and Royal Free Hospital (London, United Kingdom). This study was approved by the local ethics committee and performed according to national legislation and the declaration of Helsinki. All subjects signed informed consent forms.

Patients with HFE-HH were undergoing maintenance phlebotomies. Patients with β-thalassemia major on iron chelation medication withdrew the use of this medication for at least 4 days, to prevent bias in urinary iron. Plasma levels of deferasirox (DFX), used by six patients with β-thalassemia major, were determined as described in De Francia *et al* (28). Urinary iron levels were corrected for minimal residual iron bound to DFX in three patients. Since deferoxamine and deferiprone (used by two and one β-thalassemia major patients, respectively) have a shorter half life than DFX, we anticipate that suspending these chelators for four days eliminated plasma concentrations and subsequent urinary excretion of potential iron-chelator complexes.

Sample collection

Heparin plasma and urine were collected, aliquoted and stored at -80°C. For analysis of gluthathione s-transferase-pi-1-1 (GSTP1-1), urine was mixed and with a 10x buffer solution (1M HEPES pH 7.5, 5% bovine serum albumin, 1% sodium azide, 1% Tween-20, 10% glycerol) within 30 minutes of collection.

Laboratory analyses

Iron, total iron binding capacity (TIBC; calculated as transferrin (g/l)*25.0), creatinine, C-reactive protein (CRP), ferritin, creatinine, transferrin, and albumin analyses were performed according to routine diagnostic protocols at the Radboud Laboratory for Diagnostics at RUMC. Measurements of NTBI and labile plasma iron (LPI), the pathologically most relevant fraction of NTBI (5), were performed according Zhang *et al* (1995) (29) and Esposito *et al* (2003) (30), respectively. TSAT (the percentage of circulating transferrin that is saturated with iron (calculated as iron/TIBC*100), NTBI and LPI were used as indicators of circulating iron species (17, 18). Glomerular filtration rate was estimated (eGFR) using the modifications of diet in renal disease (MDRD) formula. Urinary iron analysis was performed by inductively coupled-plasma mass spectrometry (ICP-MS) at Maastricht University Medical Center (the Netherlands). Kidney injury marker 1 (KIM1; R&D Systems, DKM100) and GSTP1-1 (31) were measured by ELISA. All urine parameters were corrected for urinary creatinine concentration.

Statistical analysis

Data were statistically analyzed using SPSS 22 (IBM) and presented as median and interquartile range (IQR). Data were analyzed by Mann Whitney U test and non-parametric Spearman correlation coefficients. A p-value of $p<0.05$ was considered statistically significant.

Results

Clinical and laboratory characteristics

Clinical characteristics of all study participants are listed in Table 1 and results of laboratory analyses in Table 2. Plasma CRP was within reference range (<10 mg/ml) for all groups, indicating plasma iron parameters were not biased by the presence of inflammation. Subjects with systemic iron overload were older than healthy controls (50.1 ± 4.2 years vs. 37.4 ± 3.4 years, $p<0.05$), but this was not the case for patients with renal tubular dysfunction (41.9 ± 4.2 years, ns). All three groups comprised predominantly male subjects (Table 1).

Increased urinary excretion of iron, transferrin and NTBI in patients with systemic iron overload

Plasma iron, TSAT, NTBI, LPI and ferritin were increased in patients with systemic iron overload compared to healthy controls, whereas TIBC levels were decreased (ferritin $p<0.05$, others $p<0.001$; Table 2), thus confirming iron overload. Urinary iron and NTBI levels were significantly increased compared to healthy controls ($p<0.01$ and $p<0.05$, respectively; Table 2). Urinary iron levels correlated moderately with plasma TSAT ($r=0.47$, $p<0.05$; Figure 1a), but not with plasma iron or TIBC alone (Figure 1b, c). These findings indicate that urinary iron excretion could be caused by glomerular filtration of increased circulating iron levels. Moreover, we found that both urinary iron and NTBI concentrations correlated strongly with plasma ferritin ($r=0.75$, $p<0.001$, and $r=0.71$, $p<0.001$; Figure 1d, e), suggesting that urinary iron excretion could also be associated with high iron levels in renal tubuli.

We subsequently stratified patients with HFE-HH and β-thalassemia major to examine urinary iron excretion specifically in these two distinct iron overload disorders (Table 3). Patients with HFE-HH, but not with β-thalassemia major, were significantly older than healthy controls ($p<0.001$) and predominantly consisted of males, whereas both genders were equally present in β-thalassemia major. Although plasma iron levels were increased in both HFE-HH and β-thalassemia major compared to healthy controls (both $p<0.001$), β-thalassemia major patients showed a more severe increase in plasma TSAT and NTBI and decrease in TIBC (all $p<0.05$ compared to HFE-HH). Moreover, ferritin was increased in β-thalassemia major only ($p<0.001$ compared to both control and HFE-HH). These findings show that both circulating and tissue iron overload are more pronounced in patients with β-thalassemia major than in HFE-HH patients in our study cohort, confirming previous findings (18). We found urinary iron, transferrin and NTBI levels to be specifically elevated in β-thalassemia major ($p<0.001$, $p<0.05$ and $p<0.01$, respectively), where urinary NTBI strongly correlated with plasma TSAT ($r=0.75$, $p<0.05$, Figure 1f). This demonstrates that only severely elevated iron parameters, as seen in β-thalassemia major, are associated with elevated urinary iron parameters.

Increased urinary iron and transferrin excretion in patients with tubular dysfunction

Tubular dysfunction was confirmed by increased levels of PT injury marker KIM1 and DT injury marker GSTP1-1 ($p<0.01$ and $p<0.05$ respectively, compared to healthy controls; Table 2). These patients also showed increased urinary albumin levels ($p<0.05$), which were not related to eGFR (data not shown), indicating tubular proteinuria as a result of disturbed tubular reabsorption (32, 33). Despite circulating plasma iron parameters within reference range, urinary iron and transferrin, but not NTBI, were increased (both $p<0.05$). Moreover, urinary transferrin and albumin levels strongly correlated ($r=0.89$, $p<0.001$; Figure 2a), thus indicating similar glomerular filtration and tubular handling of both proteins as demonstrated before (9). Interestingly, urinary iron levels also correlated with albuminuria ($r=0.61$, $p<0.01$; Figure 2b), suggesting that insufficient tubular reabsorption contributed to urinary iron excretion.

We analyzed urinary iron and transferrin concentrations against KIM1 and GSTP1-1 to investigate the relation between iron excretion and PT and DT injury, respectively (34, 35). Whereas we found no correlation between KIM1 and urinary iron or transferrin levels (Figure 2c, d), GSTP1-1 was positively associated with both iron and transferrin ($r=0.56$, $p<0.05$, and $r=0.86$, $p<0.001$, respectively; Figure 2e, f).

Urinary iron excretion correlated with tubular injury in patients with systemic iron overload
Finally, we examined if urinary iron excretion in systemic iron overload was associated with tubular injury. Whereas GSTP1-1 levels were not affected, urinary KIM1 concentrations were increased in patients with systemic iron overload ($p<0.001$; Table 2), especially in patients with β-thalassemia major ($p<0.001$ compared to control, $p<0.05$ compared to HFE-HH; Table 3). Furthermore, urinary transferrin levels in β-thalassemia major correlated strongly with urinary KIM1 levels ($r=0.71$, $p<0.05$; Figure 3), which might indicate reduced transferrin reabsorption as a consequence of PT injury.

Discussion

Increased urinary iron concentrations have been associated with renal injury. Here, we investigated the contribution of glomerular filtration of increased circulating iron and insufficient tubular iron reabsorption to urinary iron excretion in patients with systemic iron overload and patients with renal tubular dysfunction. Our results demonstrate that both increased circulating iron levels and subsequent filtration as well as insufficient tubular reabsorption are associated with increased urinary iron concentrations and, importantly, renal injury.

Patients with tubular dysfunction were included in this study to investigate the contribution of diminished PT TBI reabsorption to urinary iron concentrations. Although PT dysfunction was confirmed by urinary albumin and transferrin excretion, only the DT injury marker correlated with urinary iron and transferrin concentrations. We propose that increased TBI levels reach the DT because of hampered PT reabsorption. Increased DT iron exposure could result in DT iron accumulation and subsequent DT injury (5). In addition, our data suggest that DT iron reabsorption capacity is not sufficient to prevent urinary iron and transferrin wasting in patients with tubular dysfunction. Therefore, both the PT and DT may contribute to urinary iron excretion during tubular dysfunction (Figure 4).

Mild systemic iron overload observed in our HFE-HH subjects did not result in urinary iron or NTBI excretion. Despite mild PT injury, tubular iron reabsorption capacity was sufficient to prevent urinary iron excretion (Figure 4). In contrast, patients with β-thalassemia major with severe iron overload, demonstrated urinary iron, transferrin and NTBI excretion. The HFE-HH patients included in our study were all undergoing maintenance phlebotomy, resulting in only mildly elevated TSAT whereas ferritin levels were not affected. Nevertheless, severe iron overload has been observed in patients with naïve HFE-HH (before start of treatment) or during depletion phlebotomy (18). Therefore, this suggests that the extent of systemic iron overload, rather than the disease pathology, determines urinary iron excretion.

Besides glomerular filtration of increased circulating iron levels, renal iron handling may have contributed to the high urinary iron excretion observed in our β-thalassemia major subjects. Increased plasma ferritin levels are indicative of high tissue iron stores, including the kidney (19). Moreover, mouse PT cells have been described to actively secrete ferritin proteins and ferritin is detected in urine in healthy volunteers (20, 36). However, iron levels in secreted ferritin are low (36) and, therefore, unlikely to largely contribute to urinary iron excretion. Alternatively, we cannot exclude passive excretion of exfoliated iron-loaded tubular epithelial cells contributed to urinary iron concentrations. Finally, PT injury observed in the β-thalassemia major patients may have influenced urinary iron and transferrin excretion. Presumably, PT injury could limit PT TBI reabsorption leading to more TBI excretion that cannot be compensated for by DT reabsorption. Altogether, urinary iron and transferrin excretion in β-thalassemia major may be resulting from filtration of increased circulating iron, enhanced iron levels in renal tubuli or PT injury (Figure 4).

Our finding of increased urinary KIM1 levels in β-thalassemia major patients adds to the rising number of reports on PT injury in β-thalassemia (37-40), and confirms previous suggestions that persistent severe systemic iron overload can lead to renal injury (41). Tubular epithelial cells can be exposed to harmful levels of iron both apically from the tubular lumen and basolaterally from the systemic circulation. Furthermore, tubular injury may also be related to use of chelation mediation (42-44). Future studies examining the etiology of renal injury during systemic iron overload are warranted, in order to prevent the emergence of this complication.

Limitations of this study include the explorative and observational design with small subject groups. Although we aimed at including subjects in age- and gender-matched groups, patients with HFE-HH were older than patients with β-thalassemia major, reflecting the average age of these patient groups in the population (45, 46). Furthermore, all study groups predominantly consisted of male subjects, with exemption of the β-thalassemia major patients. Although age above 40 years and male gender have been reported to increase levels of urinary KIM1 and albumin in healthy individuals (47, 48), this is unlikely to have largely influenced KIM1 and albumin levels in urine in β-thalassemia.

The knowledge that both filtration of systemic iron levels and tubular iron reabsorption influence urinary iron excretion has several implications. In patients with iron overload disorders prevention or reduction of tubular iron reabsorption could be used to enhance urinary iron excretion and, thus, lower the systemic iron burden and potential renal injury. The latter is also important for patients with chronic kidney disease characterized by nephropathy. Glomerular injury leads to increased protein filtration, including TBI, resulting in increased urinary iron excretion and renal iron loading (3, 9-13). In CKD animal models, increased tubular injury associated with urinary iron excretion, whereas reduction of circulating iron levels by administration of a low-iron diet or treatment with an iron chelator reduced renal tubular injury (49, 50). Also in CKD, prevention of renal iron loading by reducing tubular reabsorption could be an interesting therapeutic option.

In conclusion, our results suggest that both glomerular filtration of increased circulating iron levels and insufficient tubular iron reabsorption contribute to urinary iron excretion and are associated with renal injury.

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Conflict of Interest Statement

None declared.

Authors' Contributions

SvR performed literature search, experimental design, patient inclusion, data collection, analysis and interpretation, created figures and wrote the manuscript. AR, BB, SS, EH, SW and TN contributed to patient inclusion. EW and HR contributed to data collection and analysis. DS and RvS contributed to literature search, experimental design, data interpretation and manuscript writing. All authors read and approved the manuscript.

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References

1. Norden AG, Lapsley M, Lee PJ, Pusey CD, Scheinman SJ, Tam FW, et al. Glomerular protein sieving and implications for renal failure in Fanconi syndrome. Kidney international. 2001;60(5):1885-92.
2. Green R, Charlton R, Seftel H, Bothwell T, Mayet F, Adams B, et al. Body iron excretion in man: a collaborative study. The American journal of medicine. 1968;45(3):336-53.
3. Prinsen BH, de Sain-van der Velden MG, Kaysen GA, Straver HW, van Rijn HJ, Stellaard F, et al. Transferrin synthesis is increased in nephrotic patients insufficiently to replace urinary losses. Journal of the American Society of Nephrology : JASN. 2001;12(5):1017-25.
4. Wareing M, Ferguson CJ, Green R, Riccardi D, Smith CP. In vivo characterization of renal iron transport in the anaesthetized rat. The Journal of physiology. 2000;524 Pt 2:581-6.
5. Cabantchik ZI. Labile iron in cells and body fluids: physiology, pathology, and pharmacology. Frontiers in pharmacology. 2014;5:45.
6. Sponsel HT, Alfrey AC, Hammond WS, Durr JA, Ray C, Anderson RJ. Effect of iron on renal tubular epithelial cells. Kidney international. 1996;50(2):436-44.
7. Sheerin NS, Sacks SH, Fogazzi GB. In vitro erythrophagocytosis by renal tubular cells and tubular toxicity by haemoglobin and iron. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 1999;14(6):1391-7.
8. van Raaij SEG, Masereeuw R, Swinkels DW, van Swelm RPL. Inhibition of Nrf2 alters cell stress induced by chronic iron exposure in human proximal tubular epithelial cells. Toxicol Lett. 2018;295:179-86.
9. Howard RL, Buddington B, Alfrey AC. Urinary albumin, transferrin and iron excretion in diabetic patients. Kidney international. 1991;40(5):923-6.
10. Ellis D. Anemia in the course of the nephrotic syndrome secondary to transferrin depletion. The Journal of pediatrics. 1977;90(6):953-5.
11. Brown EA, Sampson B, Muller BR, Curtis JR. Urinary iron loss in the nephrotic syndrome--an unusual cause of iron deficiency with a note on urinary copper losses. Postgraduate medical journal. 1984;60(700):125-8.
12. Nankivell BJ, Boadle RA, Harris DC. Iron accumulation in human chronic renal disease. American journal of kidney diseases : the official journal of the National Kidney Foundation. 1992;20(6):580-4.
13. Nankivell BJ, Tay YC, Boadle RA, Harris DC. Lysosomal iron accumulation in diabetic nephropathy. Renal failure. 1994;16(3):367-81.
14. Ueda N, Baliga R, Shah SV. Role of 'catalytic' iron in an animal model of minimal change nephrotic syndrome. Kidney international. 1996;49(2):370-3.
15. Alfrey AC, Froment DH, Hammond WS. Role of iron in the tubulo-interstitial injury in nephrotoxic serum nephritis. Kidney international. 1989;36(5):753-9.
16. Alfrey AC, Hammond WS. Renal iron handling in the nephrotic syndrome. Kidney international. 1990;37(6):1409-13.
17. Brissot P, Loreal O. Iron metabolism and related genetic diseases: A cleared land, keeping mysteries. Journal of hepatology. 2016;64(2):505-15.
18. de Swart L, Hendriks JC, van der Vorm LN, Cabantchik ZI, Evans PJ, Hod EA, et al. Second international round robin for the quantification of serum non-transferrin-bound iron and labile plasma iron in patients with iron-overload disorders. Haematologica. 2016;101(1):38-45.
19. Hashemieh M, Azarkeivan A, Akhlaghpoor S, Shirkavand A, Sheibani K. T2-star (T2*) magnetic resonance imaging for assessment of kidney iron overload in thalassemic patients. Archives of Iranian medicine. 2012;15(2):91-4.
20. Sears DA, Anderson PR, Foy AL, Williams HL, Crosby WH. Urinary iron excretion and renal metabolism of hemoglobin in hemolytic diseases. Blood. 1966;28(5):708-25.
21. Choengchan N, Mantim T, Inpota P, Nacapricha D, Wilairat P, Jittangprasert P, et al. Tandem measurements of iron and creatinine by cross injection analysis with application to urine from thalassemic patients. Talanta. 2015;133:52-8.

22. Walsh JR, Fredrickson M. Serum ferritin, free erythrocyte protoporphyrin, and urinary iron excretion in patients with iron disorders. The American journal of the medical sciences. 1977;273(3):293-300.
23. Kozyraki R, Fyfe J, Verroust PJ, Jacobsen C, Dautry-Varsat A, Gburek J, et al. Megalin-dependent cubilin-mediated endocytosis is a major pathway for the apical uptake of transferrin in polarized epithelia. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(22):12491-6.
24. Ohno Y, Birn H, Christensen EI. In vivo confocal laser scanning microscopy and micropuncture in intact rat. Nephron Exp Nephrol. 2005;99(1):e17-25.
25. Thevenod F, Wolff NA. Iron transport in the kidney: implications for physiology and cadmium nephrotoxicity. Metallomics : integrated biometal science. 2016;8(1):17-42.
26. Raaij SV, Swelm RV, Bouman K, Cliteur M, Heuvel MVD, Pertijs J, et al. Tubular iron deposition and iron handling proteins in human healthy kidney and chronic kidney disease. Sci Rep. 2018;8(1):9353.
27. Langelueddecke C, Roussa E, Fenton RA, Wolff NA, Lee WK, Thevenod F. Lipocalin-2 (24p3/neutrophil gelatinase-associated lipocalin (NGAL)) receptor is expressed in distal nephron and mediates protein endocytosis. The Journal of biological chemistry. 2012;287(1):159-69.
28. De Francia S, Massano D, Piccione FM, Pirro E, Racca S, Di Carlo F, et al. A new HPLC UV validated method for therapeutic monitoring of deferasirox in thalassaemic patients. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2012;893-894:127-33.
29. Zhang D, Okada S, Kawabata T, Yasuda T. An improved simple colorimetric method for quantitation of non-transferrin-bound iron in serum. Biochem Mol Biol Int. 1995;35(3):635-41.
30. Esposito BP, Breuer W, Sirankapracha P, Pootrakul P, Hershko C, Cabantchik ZI. Labile plasma iron in iron overload: redox activity and susceptibility to chelation. Blood. 2003;102(7):2670-7.
31. Mulder TP, Peters WH, Wobbes T, Witteman BJ, Jansen JB. Measurement of glutathione S-transferase P1-1 in plasma: pitfalls and significance of screening and follow-up of patients with gastrointestinal carcinoma. Cancer. 1997;80(5):873-80.
32. van Berkel Y, Ludwig M, van Wijk JAE, Bokenkamp A. Proteinuria in Dent disease: a review of the literature. Pediatric nephrology. 2017;32(10):1851-9.
33. Norden AG, Scheinman SJ, Deschodt-Lanckman MM, Lapsley M, Nortier JL, Thakker RV, et al. Tubular proteinuria defined by a study of Dent's (CLCN5 mutation) and other tubular diseases. Kidney international. 2000;57(1):240-9.
34. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. The Journal of biological chemistry. 1998;273(7):4135-42.
35. McMahon BA, Koyner JL, Murray PT. Urinary glutathione S-transferases in the pathogenesis and diagnostic evaluation of acute kidney injury following cardiac surgery: a critical review. Curr Opin Crit Care. 2010;16(6):550-5.
36. Cohen LA, Gutierrez L, Weiss A, Leichtmann-Bardoogo Y, Zhang DL, Crooks DR, et al. Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. Blood. 2010;116(9):1574-84.
37. Hashemieh M, Radfar M, Azarkeivan A, Hosseini Tabatabaei SMT, Nikbakht S, Yaseri M, et al. Renal Hemosiderosis among Iranian Transfusion Dependent beta-Thalassemia Major Patients. Int J Hematol Oncol Stem Cell Res. 2017;11(2):133-8.
38. ElAlfy MS, Khalil Elsherif NH, Ebeid FSE, Ismail EAR, Ahmed KA, Darwish YW, et al. Renal iron deposition by magnetic resonance imaging in pediatric beta-thalassemia major patients: Relation to renal biomarkers, total body iron and chelation therapy. Eur J Radiol. 2018;103:65-70.
39. Annayev A, Karakas Z, Karaman S, Yalciner A, Yilmaz A, Emre S. Glomerular and Tubular Functions in Children and Adults with Transfusion-Dependent Thalassemia. Turk J Haematol. 2018;35(1):66-70.

40. Deveci B, Kurtoglu A, Kurtoglu E, Salim O, Toptas T. Documentation of renal glomerular and tubular impairment and glomerular hyperfiltration in multitransfused patients with beta thalassemia. Ann Hematol. 2016;95(3):375-81.
41. Bhandari S, Galanello R. Renal aspects of thalassaemia a changing paradigm. European journal of haematology. 2012;89(3):187-97.
42. Chuang GT, Tsai IJ, Tsau YK, Lu MY. Transfusion-dependent thalassaemic patients with renal Fanconi syndrome due to deferasirox use. Nephrology (Carlton). 2015;20(12):931-5.
43. Shah L, Powell JL, Zaritsky JJ. A case of Fanconi syndrome due to a deferasirox overdose and a trial of plasmapheresis. J Clin Pharm Ther. 2017;42(5):634-7.
44. Cianciulli P, Sollecito D, Sorrentino F, Forte L, Gilardi E, Massa A, et al. Early detection of nephrotoxic effects in thalassemic patients receiving desferrioxamine therapy. Kidney international. 1994;46(2):467-70.
45. Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. Lancet. 2018;391(10116):155-67.
46. European Association For The Study Of The L. EASL clinical practice guidelines for HFE hemochromatosis. Journal of hepatology. 2010;53(1):3-22.
47. Pennemans V, Rigo JM, Faes C, Reynders C, Penders J, Swennen Q. Establishment of reference values for novel urinary biomarkers for renal damage in the healthy population: are age and gender an issue? Clin Chem Lab Med. 2013;51(9):1795-802.
48. Jones CA, Francis ME, Eberhardt MS, Chavers B, Coresh J, Engelgau M, et al. Microalbuminuria in the US population: third National Health and Nutrition Examination Survey. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2002;39(3):445-59.
49. Ikeda Y, Enomoto H, Tajima S, Izawa-Ishizawa Y, Kihira Y, Ishizawa K, et al. Dietary iron restriction inhibits progression of diabetic nephropathy in db/db mice. American journal of physiology Renal physiology. 2013;304(7):F1028-36.
50. Ikeda Y, Ozono I, Tajima S, Imao M, Horinouchi Y, Izawa-Ishizawa Y, et al. Iron chelation by deferoxamine prevents renal interstitial fibrosis in mice with unilateral ureteral obstruction. PloS one. 2014;9(2):e89355.

Tables

Table 1: Subject characteristics

	Healthy controls	Renal tubular dysfunction	Systemic iron overload
Subjects (n)	20	18	20
Age (years)	37.4 ± 3.4	41.9 ± 4.2	50.1 ± 4.2*
Gender (M/F)	12/8	17/1	14/6
Type of disorder	-	Cystinosis, n=2 Dent's disease, n=3 Fanconi syndrome, n=3 Nephronophthisis, n=1 Tubulo-interstitial nephritis, n=1 Wilson's disease, n=1 Secondary to chemotherapy, n=5 Secondary to lithium-induced nephrogenic diabetes insipidus, n=1 Secondary to unknown cause, n=1	β-thalassemia major, n=9 Diamond Blackfan anemia, n=1 HFE-related hereditary hemochromatosis, n=10

Age presented as mean ± standard error of the mean (SEM). *, $p<0.05$, compared to healthy controls by Mann Whitney U test.
F, female; M, male.

Table 2: Results of laboratory analyses in the 3 study groups

		Healthy controls	Renal tubular dysfunction	Systemic iron overload
Plasma	Iron (µmol/l)	16.0 (12.7 – 18.7)	15.0 (12.5 – 21.5)	30.5 (27.5 – 39.5)***
	TIBC (µmol/l)	64.0 (57.2 – 73.0)	60.0 (53.7 – 74.7)	44.5 (41.2 – 48.0)***
	TSAT (%)	24.5 (20.1 – 30.8)	24.4 (17.2 – 33.6)	74.9 (62.5 – 89.7)***
	Ferritin (µg/l)	121.0 (68.0 – 178.5)	179.5 (38.5 – 269.8)	371.0 (111.0 – 906.8)*
	NTBI (µmol/l)	0.23 (0.23 – 0.23)	0.23 (0.23 – 0.23)	1.22 (0.72 – 2.32)***
	LPI (µmol/l)	0.11 (0.10 – 0.14)	0.15 (0.11 – 0.22)	0.24 (0.22 – 0.31)***
	CRP (mg/ml)	0.5 (0.5 – 0.5)	1.5 (0.5 -2.2)	1.0 (0.5 – 2.0)
Urine	Iron (µmol/g creatinine)	0.03 (0.02 – 0.07)	0.09 (0.03 – 0.20)*	0.21 (0.05 – 2.39)**
	Transferrin (mg/g creatinine)	1.0 (0.6 – 2.7)	3.0 (0.9 – 11.8)*	1.1 (0.6 – 10.8)
	NTBI (µmol/g creatinine)	0.4 (0.2 – 0.7)	0.6 (0.4 – 1.6)	0.5 (0.2 – 1.7)*
	Albumin (mg/g creatinine)	3.8 (2.8 – 5.7)	27.5 (9.2 – 168.5)***	13.1 (7.1 – 283.8)***
	KIM1 (ng/mmol creatinine)	0.3 (0.1 – 0.5)	0.7 (0.4 – 0.8)**	0.9 (0.6 – 2.2)***
	GSTP1-1 (ng/mmol creatinine)	3.1 (1.8 – 6.2)	7.6 (2.1 – 65.6)*	2.6 (0.9 – 9.3)
eGFR		90.0 (67.8 – 100.8)	52.4 (34.6 – 80.1)***	76.8 (64.0 – 109.5)

(ml/min/1.73m^2)			

Results presented as median (interquartile range (IQR)). *, p<0.05; **, p<0.01; ***, p<0.001 compared to healthy controls by Mann Whitney U test. eGFR calculated by MDRD formula.
CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GSTP1-1, gluthathione s-transferase-pi-1-1; KIM1, kidney injury marker 1; LPI, labile plasma iron; NTBI, non-transferrin-bound iron; TIBC, total iron binding capacity; TSAT, transferrin saturation.

Table 3: Laboratory results in subjects with HFE-related hereditary hemochromatosis and β-thalassemia major

		HFE-related hereditary hemochromatosis	**β-thalassemia major**
Subjects (n)		10	9
Age (y)		63.8 ± 3.7***	37.4 ± 4.5
Gender (M/F)		9/1	5/4
Plasma	Iron (µmol/l)	30.0 (26.5 – 34.7)***	31.0 (27.5 – 42.5)***
	TIBC (µmol/l)	46.0 (43.0 – 48.2)***	42.0 (35.0 – 46.5)***, #
	TSAT (%)	66.9 (58.7 – 77.6)***	90.2 (71.9 – 92.6)***, #
	Ferritin (µg/l)	113.0 (85.0 – 185.3)	958.0 (383.0 – 1504.0)***, ###
	NTBI (µmol/l)	0.94 (0.43 – 1.25)***	2.31 (1.20 – 2.54)***, #
	LPI (µmol/l)	0.23 (0.19 – 0.24)***	0.31 (0.24 – 0.46)***, #
Urine	Iron (µmol/g creatinine)	0.1 (0.02 – 0.09)	2.4 (1.0 – 3.8)***, ###
	Transferrin (mg/g creatinine)	0.8 (0.6 – 1.3)	2.8 (1.1 – 26.4)*, #
	NTBI (µmol/g creatinine)	0.2 (0.1 – 0.4)	1.5 (0.7 – 2.5)**, ###
	Albumin (mg/g creatinine)	8.4 (5.2 – 13.5)**	23.9 (11.6 – 705.6)***, #
	KIM1 (ng/mmol creatinine)	0.9 (0.5 – 1.0)**	2.2 (0.9 – 3.4)***, #
	GSTP1-1 (ng/mmol creatinine)	1.6 (0.7 – 8.8)	5.9 (1.7 – 10.1)
eGFR (ml/min/1.73m^2)		76.8 (62.8 – 99.3)	76.3 (62.1 – 129.0)

Results as median (interquartile range (IQR)). *, p<0.05; **, p<0.01; ***, p<0.001 compared to healthy controls (Table 2); #, p<0.05; ##, p<0.01; ###, p<0.001 compared to hereditary hemochromatosis by Mann Whitney U test. eGFR calculated by MDRD formula.
eGFR, estimated glomerular filtration rate; GSTP1-1, gluthathione s-transferase-pi-1-1; KIM1, kidney injury marker 1; LPI, labile plasma iron; NTBI, non-transferrin-bound iron; TIBC, total iron binding capacity; TSAT, transferrin saturation.

Legends to figures

Figure 1: Urinary iron and non-transferrin-bound iron (NTBI) correlate with plasma iron parameters in systemic iron overload

Correlation between urinary iron excretion and plasma transferrin saturation (TSAT) (a), iron (b), total iron binding capacity (TIBC) (c) and ferritin (d); urinary NTBI and plasma ferritin in patients with systemic iron overload; and urinary NTBI and plasma TSAT in patients with β-thalassemia major (f). Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value or not significant (ns)).

Figure 2: Urinary iron and transferrin correlate with urinary kidney parameters in tubular dysfunction

Correlation between urinary transferrin and iron with urinary albumin (a, b), proximal tubular injury marker kidney injury marker 1 (KIM1) (c, d) and distal tubular injury marker gluthathione s-transferase-pi-1-1 (GSTP1-1) (e, f) in patients with tubular dysfunction. Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value or not significant (ns)).

Figure 3: Urinary transferrin correlates with urinary proximal tubular injury in β-thalassemia major

Correlation between urinary transferrin and proximal tubular injury marker kidney injury marker 1 (KIM1) in patients with β-thalassemia major. Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value or not significant (ns)).

Figure 4: Proposed mechanism of iron reabsorption in the kidney in health, tubular dysfunction and systemic iron overload.

In health (left panel), circulating transferrin-bound iron (TBI) is filtered into the renal tubular lumen by the glomerulus, and subsequently is completely reabsorbed. This is predominantly done by proximal tubules (PTs) and only to a minor extent in distal tubules (DTs). During tubular dysfunction (second left panel), similar TBI levels are filtered into the tubular lumen, but these are reabsorbed to a lower extent by PTs as a result of PT injury (indicated by lightning sign). Consequently, DTs reabsorb larger amounts of TBI, but, as a result of DT injury, iron (in red) and transferrin (in blue) are excreted in urine. In mild systemic iron overload (second right panel), increased circulating TBI and non-transferrin-bound iron (NTBI) are filtered into the tubular lumen. Reabsorption in PTs, despite mild injury, and DTs prevent iron and transferrin excretion. In severe systemic iron overload (right panel), TBI and NTBI are filtered into the tubular lumen in even larger concentrations, but limitedly reabsorbed by PTs as a result of increased intracellular iron levels and PT injury. Iron reabsorption capacity in DTs is not sufficient to prevent iron and transferrin excretion in urine.